

Enhanced Production of *Pyrenophora tritici-repentis* Conidial Suspensions

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ABSTRACT

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A technique that yields conidial suspensions of *Pyrenophora tritici-repentis* nearly free of other propagules involves growing the fungus in potato-dextrose broth, followed by removal, comminution, and inoculation into liquid clarified V8 juice agar (CV8). After 48 hr of growth in the dark, the fungus is exposed to alternating periods of light and dark that stimulate the production of conidiophores and conidia in a lawn covering the entire surface of the colony. In two tests using 24 isolates of *P. tritici-repentis*, inoculum suspensions were obtained with an average of 1,386 and 1,692 propagules (conidia, conidiophores, and hyphal fragments) per milliliter, in which 92 and 100%, respectively, of the propagules were conidia. In a third test, the use of a directed stream of water to remove inoculum resulted in a suspension in which 98% of the total propagules were conidia. Use of a glass microscope slide or a rubber spatula to scrape inoculum from agar surfaces resulted in suspensions in which only 67% of the total propagules were conidia. A fourth test demonstrated that conidial lawns resulted in inoculum yields two to three times greater than those obtained from conidial rings formed on the periphery of fungal colonies grown on CV8 medium. The technique enhances conidial yield and facilitates the production of conidial suspensions nearly free of other *P. tritici-repentis* propagules. The technique will promote more precise and repeatable genetic and epidemiological studies of tan spot of wheat.

Pyrenophora tritici-repentis (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker), the causal agent of tan spot of wheat (*Triticum aestivum* L.), has been the focus of genetic (9,10,12), epidemiological (6,11,16), and other studies. Although in vitro production of conidia by *P. tritici-repentis* has been reported (13), obtaining conidial suspensions unadulterated with other infective propagules (OIP), i.e., conidiophores and hyphal fragments, is difficult. The basic technique previously used to produce conidia involves growing the fungus sequentially on potato-dextrose agar in darkness, followed by growth on V8 juice agar exposed to alternating periods of light and dark (14). Exposure to light induces *P. tritici-repentis* to produce conidiophores near the perimeter of the fungal colony. A subsequent dark period induces the fungus to produce conidia in a ring near the fungal colony periphery. This results in rings of conidia associated with the period(s) of darkness.

Conidia produced by cultures usually are removed from agar surfaces by flooding petri plates with water and gently scraping the colony surface with a rubber policeman, metal spatula, glass slide, bent-glass rod, or wire loop (5,8,15,16,18). Although these techniques are

used to produce conidial suspensions, they are not efficient in producing conidia. For example, the entire surface of the V8 juice agar is not used for conidial production, and several periods of alternating light and dark are required to produce multiple conidial bands. Most important, removal of conidia from agar surfaces results in suspensions of inocula composed of a conglomerate of conidia, conidiophores, and aerial hyphae (1-3). Quantifying inocula of such a conglomerate is difficult, and reproducing the composition (conidia, conidiophores, and hyphal fragments) of inocula between experiments is nearly impossible. This paper describes a technique resulting in high yields of conidia that are produced in lawns over the entire surface of V8 juice agar in petri dishes and in conidial suspensions that are relatively free of OIP (4).

MATERIALS AND METHODS

Twenty-four *P. tritici-repentis* isolates obtained from three asci (eight single-ascospore isolates [SAI] per ascus) were used in the first two tests. Each set of eight SAI was obtained from pseudothecia that formed on straw infested with *P. tritici-repentis* collected during the summer of 1991 from wheat fields near Altus, Guymon, and Braman, Oklahoma. Isolates were characterized for growth and appearance in vitro and stored for later use in cryogenic tubes with one drop of 10% dimethyl sulfoxide at -70 C (6). The third test consisted of three of the SAI (one each from Altus, Braman, and Guymon) used in the first two tests and three single conidial isolates

obtained from infested straw collected from wheat fields near Clinton, Goltry, and Ponca City, Oklahoma. The three SAI used in the third experiment were used in a fourth test.

Production of conidial inoculum.

Isolates of *P. tritici-repentis* were grown on freshly prepared potato-dextrose agar (PDA; 200 g of potatoes, 20 g of dextrose, and 15 g of agar per liter of medium). After 3 days of growth on PDA at 21 C in darkness, five plugs (5 mm diameter) were excised from the periphery of each colony and transferred to 100 ml of potato-dextrose broth (PDB; PDA without agar) in a 250-ml Erlenmeyer flask. Flasks were incubated at room temperature (20-25 C) and exposed to room lighting for 12-15 hr on an orbital shaker table at 1,200 rpm. After 5 days, the mycelial mass was poured onto a sterile nylon screen and squeezed with a sterile microspoon to remove excess PDB. Then, 10 g of fresh weight from the mycelial mass was placed in 20 ml of sterile distilled water in a 50-ml beaker and comminuted for 30 sec at 13,500 rpm using a Tissumizer Mark II (Tekmar Co., Cincinnati, OH) with the S25N-10G dispersing tool. Following comminution of each isolate, the dispersing tool was sequentially rinsed once in 1% sodium hypochlorite solution, once in 95% ethanol solution, and twice in sterile distilled water.

Following comminution, each isolate was set aside until all were prepared within approximately 20 min. First, 2 ml of the comminuted mycelial suspension was added to 100 ml of liquid clarified V8 juice agar (CV8; 3 g of CaCO₃ and 150 ml of V8 juice centrifuged for 10 min at 2,000 rpm, plus 15 g of agar per liter of medium) in a 250-ml Erlenmeyer flask maintained at 48 C. The flask was swirled to distribute the comminute in the CV8, which was poured into plastic petri dishes (100 × 15 mm). Four plates (replications) were poured for each *P. tritici-repentis* isolate. After the plates were incubated for 48 hr at 24 C in darkness, aerial hyphae were appressed to the agar surface using three drops (0.04 ml per drop) of sterile distilled water and a sterile bent-glass rod. Plates were incubated for 24 hr in light (51 μE·m⁻²·s⁻¹) and then for 24 hr in darkness at 24 C to induce conidiophore and conidia formation. Following the final incubation period, plates were examined with a stereomicroscope to verify conidial production.

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Conidia were removed from the agar surface with a stream of distilled water (containing one drop of Tween 20 per 100 ml) from a plastic wash bottle. This was done carefully to minimize disruption of the agar medium surface. Each plate was washed with 100 ml of solution, which was sufficient to remove conidia. Plates were examined with a stereomicroscope after washing to determine the effectiveness of conidia removal. One drop of safranin stain was added to a nematode counting dish prior to addition of the conidial suspension to facilitate counting of conidia with a stereomicroscope (7). After conidia were counted, 1 ml of suspension was pipetted onto water agar and observed after 2 hr to determine the percent germination of conidia. Concentrations of conidia and OIP in the suspension were determined, and the percentages of conidial propagules were derived by dividing the number of conidia per milliliter by the sum of conidia and OIP per milliliter. The percent values were transformed to arcsine-square root values, analyses were conducted using the SAS GLM procedure, and mean separations were computed following the Waller-Duncan Bayesian *k*-ratio *t* test option with a specified *k*-ratio of 100 to compute the minimum significant difference value (17). The statistical design was a ran-

domized complete block with four replications.

Procedures in a repeated test were the same as in the first test except that 2 ml of comminute was added immediately into the liquid CV8. This was done to minimize the time isolates were left in sterile distilled water. Determination of conidia and OIP concentration and statistical analyses were conducted as in test 1.

Removal of inoculum. Three methods of removing inoculum from the surface of agar medium in petri plates were evaluated using six *P. tritici-repentis* isolates in two experiments. The experimental design was a 3 × 6 factorial with four replications; each test was conducted twice. Procedures in the first test were the same as those in the second test except that an incubation temperature of 21 C was used instead of 24 C to improve conidial production (14).

The first method involved washing inoculum from the surface of petri plates with a directed stream of distilled water with one drop Tween 20 per 100 ml as described previously. The second method followed a reported technique (16) in which 10 ml of water, with one drop Tween 20 per 100 ml, was added to the surface of a *P. tritici-repentis* colony growing on CV8. Inoculum was gently dislodged by scraping the surface of the

colony with a rubber spatula. Inoculum was poured into a 250-ml Erlenmeyer flask, and the plate was rinsed with an additional 10 ml of distilled water with one drop Tween 20 per 100 ml, which was added to the 250-ml flask. The final volume was adjusted to 100 ml, and inoculum composition was determined. The third method of inoculum removal was based on a previous report (5) and is similar to the rubber spatula method except that a glass microscope slide was used to gently scrape the surface of the colony. The volumes used per plate for the initial scraping and rinse were the same as those used in the rubber spatula method. The final volume was adjusted to 100 ml, and inoculum composition was determined. The percent conidia values were transformed to arcsine-square root values, and analyses were conducted by the SAS GLM procedure. Mean separations for method of inoculum removal were computed following the Waller-Duncan Bayesian *k*-ratio *t* test option with a specified *k*-ratio of 100 to compute the minimum significant difference value (17).

Conidial inoculum yield. The yields of inoculum resulting from two methods of conidial production were evaluated using *P. tritici-repentis* isolates 1, 11, and 22 (Table 1) in two experiments. The experimental design of each test was a 2 × 3 factorial with four replications; each test was conducted twice.

Two methods were used to produce conidia. One method followed the procedures described earlier resulting in conidial lawns grown over the entire surface of CV8 medium. The second method followed a technique in which more than one alternating light and dark period (2,9) was used to produce conidia on CV8. After 3 days of growth on PDA at 21 C in darkness, four plugs (5 mm diameter) were excised from the periphery of each colony and transferred to the surface of CV8 medium in petri dishes (replications). After the plates were incubated for 72 hr at 21 C in darkness, aerial hyphae were appressed to the agar surface using three drops (0.04 ml per drop) of sterile distilled water and a sterile bent-glass rod. Plates were incubated for 24 hr in light (51 μE·m⁻²·s⁻¹) and then for 24 hr in darkness at 21 C to induce conidiophore and conidia formation. Conidia were produced so that inoculum from each method was harvested at the same time.

In a second test, each method of conidial production was performed as described except that after hyphae were appressed to the agar, plates were incubated for 24 hr in light followed by 24 hr in darkness, 24 hr in light, and 24 hr in darkness. This resulted in the formation of two conidial rings on the agar surface.

In both experiments, conidia were removed from agar surfaces with a

Table 1. Asexual inoculum production and composition by isolates of *Pyrenophora tritici-repentis* produced by directed washing of cultures grown in clarified V8 juice medium

Isolate ^y	Inoculum composition per milliliter					
	Test 1			Test 2		
	Conidia	OIP ^w	Conidia ^x (%)	Conidia	OIP	Conidia (%)
1	913 bc ^v	6 de	99.3	1,676 a	1	99.9
2	852 b-e	14 c-e	98.7	1,690 a	2	99.9
3	857 b-e	7 de	99.4	1,379 ab	4	99.7
4	650 b-g	9 de	98.7	750 de	2	99.8
5	781 b-f	6 de	99.2	1,272 a-c	3	99.8
6	747 b-f	3 de	99.8	1,530 ab	1	99.9
7	902 b-d	9 de	99.3	1,540 ab	4	99.7
8	572 e-g	5 de	99.4	1,209 bc	1	99.9
9	963 b	28 bc	97.4	506 d-g	6	98.3
10	500 f-h	26 bc	92.5	405 e-g	2	99.2
11	1,320 a	46 a	96.7	690 d-f	2	99.8
12	570 e-g	13 c-e	97.6	632 d-g	4	99.3
13	572 e-g	32 ab	95.7	410 e-g	3	99.4
14	476 f-h	6 de	99.1	417 e-g	4	98.5
15	580 d-g	15 c-e	97.7	682 d-f	4	99.3
16	582 d-g	9 de	98.9	845 cd	6	99.4
17 ^z	0 j	0 e	...	206 g	3	99.3
18	25 j	1 de	92.3	364 e-g	4	99.3
19	215 h-j	6 de	99.1	313 fg	5	95.6
20	383 g-i	9 de	99.0	506 d-g	7	98.1
21	92 ij	0 e	100.0	431 d-g	8	97.5
22	508 f-h	4 de	99.2	600 d-g	2	99.5
23	131 ij	1 de	99.8	624 d-g	3	99.7
24	605 c-g	17 b-d	98.1	361 e-g	2	99.5

^vIsolates 1-8, 9-16, and 17-24 represent single-ascospore isolates from three asci from Altus, Guymon, and Braman, Oklahoma, respectively.

^wOther infective propagules, i.e., conidiophores and hyphal fragments.

^xData were transformed to arcsine-square root values for analysis; actual percentages are shown.

^yMeans within columns followed by the same letter are not significantly different according to the Waller-Duncan Bayesian *k*-ratio *t* test with a specified *k*-ratio of 100. Means in columns without letters are not significantly different.

^zOmitted from the analysis of percent conidia in Test 1.

stream of distilled water, and volumes were adjusted to 100 ml as described previously. Conidia and OIP were counted in a nematode counting dish, and yields of conidia and OIP per petri dish for each method of inoculum production were determined by multiplying the counts by 100. Sporulation density was determined by dividing the number of conidia by the surface area of the agar medium in a 100 × 15 mm petri dish. Both experiments were conducted twice, and separate analyses were made following the SAS ANOVA procedure (17). Mean separations were conducted using an LSD computed at $P = 0.05$.

RESULTS

All isolates used in the four tests were dark green when grown on PDA and typically produced conidiophores and conidia at their colony edges when grown on CV8 as described previously (7). In all tests, the viability of conidia, as indicated by germination on the surface of water agar, was greater than 98%.

Production of conidial inoculum. The statistical analyses with tests one and two indicated there was an isolate × test interaction for conidial production. Hence, data from tests one and two were analyzed and presented separately (Table 1).

In tests one and two, significant differences in conidial production among isolates were detected. The SAI from Altus (isolates 1–8) and Braman (isolates 17–24) produced more conidia in the second test. With the exception of isolate 11, production by SAI from Guymon (isolates 9–16) was stable over tests.

In tests one and two, there were no significant differences among isolates for the percentage of conidia in suspension; this ranged from 92 to 100% in test 1 and from 95–99% in test 2 (Table 1). There were significant differences among isolates with regard to OIP in the first test but not in the second. In both tests, suspensions of conidia nearly free of OIP were obtained for all isolates.

Methods of inoculum removal. The analysis indicated that there were significant differences in inoculum yields between tests, among inoculum removal methods, and among isolates for number of conidia, number of OIP, and percent conidia per milliliter (Table 2). For each of these variables, the inoculum removal method explained the greatest magnitude of the variation observed. The analysis also indicated there was no significant interaction between inoculum removal method and *P. tritici-repentis* isolate. Mean yields of inocula were higher in the second test. Data were presented from the tests as means for the inoculum removal method averaged over the six *P. tritici-repentis* isolates to show the effects of using different methods of inoculum removal (Table 3). The percent conidia of the total propagules in suspension was significantly higher when a directed stream of water from a plastic wash bottle was used than when the other two methods were used (Table 3). The percent conidia was identical with the spatula and microscope slide methods of removal.

Conidial inoculum yield. In both experiments comparing the methods of inoculum production, significant differences were observed among isolates and between methods of inoculum production. Significant method × isolate interactions were observed in both experiments as well. There were no significant differences between tests that were repeated.

Mean yield of conidia induced by exposure of *P. tritici-repentis* isolates to one photoperiod was significantly higher when produced over the entire surface of the medium in petri dishes in conidial lawns (Table 4); the yield of conidia obtained by growing conidia in a ring was approximately one-third that of the yield obtained from conidial lawns. Mean yield of conidia induced by exposure of isolates to two photoperiods was also significantly higher when produced in a conidial lawn. Again,

spore density of conidia produced in a lawn was significantly greater than that of conidia grown in two conidial rings.

DISCUSSION

We have described a technique that consistently results in suspensions of conidia relatively unadulterated with OIP when using an array of SAI and single-conidium isolates of *P. tritici-repentis* collected from different geographic regions of Oklahoma. By comparison, scraping *P. tritici-repentis* colonies on agar surfaces with an instrument such as a rubber spatula or a glass microscope slide results in conidial suspensions that contain a high proportion of OIP. We specifically demonstrated that using a directed stream of water to remove conidia results in suspensions with significantly lower concentrations of OIP. Suspensions with high numbers of OIP may be appropriate in some studies but may not accurately reflect natural inoculum when used in genetic and epidemiological studies of tan spot. Additionally, production of conidial suspensions free of OIP minimizes conglomeration of conidiophores, hyphal fragments, and conidia that typically occurs when large amounts of OIP are present in the suspension (*unpublished*). Also, the concentration of conidia in a suspension nearly free of OIP is easily adjusted by collecting conidia on Nitex nylon screen (25 μm) with resuspension in a smaller volume of water. Conidia readily become resuspended rather than conglomerate with the OIP because the amount of OIP is extremely low.

The technique also allows production of conidia that are ontogenetically

Table 2. Analysis of variance for the comparison of several techniques used to harvest inoculum of *Pyrenophora tritici-repentis* from the surface of clarified V8 juice medium

Source	df	Mean square values		
		Conidia (no./ml)	OIP ^y (no./ml)	Conidia ^w (%/ml)
		× 10 ⁸		
Test	1	1,313.33***	276.36*	0.1335**
Rep (test)	6	85.94 NS	28.93 NS	0.0099*
Method ^y	2	2,905.43**	2,549.13**	1.4919**
Isolate ^z	5	254.34 NS	35.86 NS	0.0104*
Method × isolate	10	5.78 NS	10.40 NS	0.0034 NS
Error	119	77.95	17.03	0.0045

^wOther infective propagules, i.e., conidiophores and hyphal fragments.

^yValues were transformed by the arcsine-square root method for analysis of variance.

*NS = not significant, * = significant at $P \leq 0.05$, and ** = significant at $P \leq 0.01$.

^zInoculum was removed from cultures by scraping with a rubber spatula or a glass microscope slide or by washing with a directed stream of water from a plastic wash bottle.

^zIsolates were collected from wheat fields near Altus, Braman, Clinton, Goltry, Guymon, and Ponca City, Oklahoma.

Table 3. Quantification of inoculum composition using three harvest methods evaluated over two tests utilizing six isolates of *Pyrenophora tritici-repentis* grown in clarified V8 juice medium in a factorial design

Method ^w	Inoculum composition per milliliter		Conidia ^y (%)
	Conidia	OIP ^x	
Directed washing	4,033 a ^z	68 c	98.2 a
Rubber spatula	2,900 b	1,419 a	67.7 b
Microscope slide	2,542 c	1,217 b	67.7 b

^wInoculum was removed by scraping with a rubber spatula or a glass microscope slide or by washing with a directed stream of water from a plastic wash bottle.

^xOther infective propagules, i.e., conidiophores and hyphal fragments.

^yData were transformed to arcsine-square root values for analysis; actual percentages are shown.

^zMeans within columns followed by the same letter are not significantly different according to the Waller-Duncan Bayesian k -ratio t test with a specified k -ratio of 100 to compute the minimum significant difference.

Table 4. Quantification of mean inoculum yield and conidia density of *Pyrenophora tritici-repentis* per petri dish of clarified V8 juice medium comparing two inoculum production methods in tests with one or two photoperiods

Test ^y Method	Harvested inoculum composition ^w		Conidia ^y (no./mm ²)
	× 10 ²		
	Conidia	OIP ^x	
One photoperiod			
Conidial lawn	3,395 a ^z	93 a	56.8 a
Conidial ring	955 b	81 a	16.1 b
Two photoperiods			
Conidial lawn	2,977 a	211 a	50.1 a
Conidial rings	1,582 b	206 a	26.6 b

^yEach test was conducted twice using isolates 1, 11, and 22 (Table 1) in a factorial design with four replications.

^wInoculum was removed by washing with a directed stream of water from a plastic wash bottle.

^xOther infective propagules, i.e., conidiophores and hyphal fragments.

^ySpore density per unit surface area of medium was derived by dividing the yield of conidia by the surface area of medium in a 100 × 15 mm petri dish.

^zMean propagule yields per petri dish within columns of each test followed by the same letter are not significantly different according to the least significant difference test ($P = 0.05$).

similar, in contrast to those produced from multiple conidial rings. Additionally, we have demonstrated that inoculum yields can be significantly increased by utilizing the entire surface of agar medium in petri dishes to produce conidial lawns. We used an excessive volume of water to remove conidia from the surface of agar medium mainly to standardize our procedures and simplify counting conidial concentrations. Lesser volumes of water could be used to remove conidia from agar surfaces and thus increase the concentration of conidia in the suspensions. Furthermore, this technique may be adaptable for inoculum preparation using fungi in similar or related groups.

We should note that the technique did not consistently differentiate the abilities of our *P. tritici-repentis* isolates to produce conidia, and other methods may be more appropriate for this determination. However, our experiments were specifically designed to demonstrate a technique that efficiently produces higher yields of *P. tritici-repentis* conidia and conidial suspensions nearly free of OIP, rather than to differentiate the

abilities of fungal isolates to produce conidia. We feel that the reported technique will facilitate precise, quantifiable, and repeatable studies that address the infection kinetics of *P. tritici-repentis* conidia as well as other genetic and epidemiological studies where precise, uniform inoculum of *P. tritici-repentis* conidia is required.

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